

PRVPATENT- OCH REGISTRERINGSVERKET
Patentavdelningen**Intyg
Certificate**

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.



(71) Sökande SP Sveriges Provnings och Forskningsinstitut AB,
Applicant (s) Borås SE

(21) Patentansökningsnummer 0201705-1
Patent application number

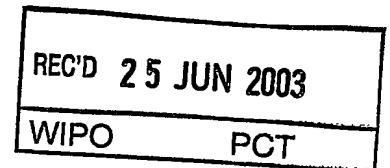
(86) Ingivningsdatum 2002-06-05
Date of filing

Stockholm, 2003-06-12

För Patent- och registreringsverket
For the Patent- and Registration Office

Sonia André
Sonia André

Avgift
Fee



PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

APPLICANT: SVERIGES PROVNINGS- OCH
FORSKNINGSINSTITUT AB

TITLE: IMPROVED ANALYSIS

5

The invention refers to a new technique for global measurements of subcellular dynamics of gene expression, proteins, metabolites etc, the spatial distribution of at least one chemical substance retained by a biological matter being analyzed.

Cellular reactions against allogenic materials involve the production of signal mediators in connection with contact with different materials and drugs.

For example, the blood reaction to foreign materials may engage several major defense systems, e.g. the coagulation cascade, the complement system, fibrinolysis, the kinins, platelet derived growth factors, platelet chemokines, and leukocyte derived factors, like prostaglandins, lipid peroxidation products or ceramides. Attempts to measure blood reactions to materials by choosing one of these factors will always meet with the possibility that other factors may be more important. Methods available today for measuring cell reactions comprise immunocytochemistry and the like, one pre-determined substance at a time being detected.

In connection with for example proteomics it is also desirable to be able to apply general global measurements, whereby a large number of components, for example proteins, can be simultaneously detected in one sample only. Global measurements can explain how proteins, nucleic acids, and small molecules interact with each other to form networks or modules that carry out specific functions. Today, such measurements are integrated, i.e. the measurements are performed in liquid media or cell suspensions, large volumes being required with accompanying complicated separation techniques.

Thus there is a strong demand in the rapidly
advancing fields of gene expression acquisition techno-
logies, gene expression data analysis, functional analysis
of biological control systems, proteomics, modelling and
5 analysis of kinetic networks, metabolomics, signal trans-
duction, morphogenesis, molecular neurobiology, etc, for a
method, whereby it is possible to measure several factors
simultaneously, rather than by studying the detailed
behaviour of single components. Methods for global measure-
10 ments on individual cells, including subcellular levels,
are not available today.

According to the invention, a method is provided for
analyzing the spatial distribution of at least one chemical
substance retained by a biological matter. The chemical
15 substance should mainly comprise organic material, which
for example can comprise a lipid, an amino acid, a peptide,
a protein, a carbohydrate, a nucleotide, a transmitter
substance, a drug, or a targeting molecule. The biological
matter can for example comprise cells, tissue, virus, body
20 liquid, or biological molecules. Thus, the chemical sub-
stance retained by the biological matter can be located
within or on the same.

In order to determine the spatial distribution of the
chemical substance, a targeting molecule could be arranged
25 to bind to or react with specific targeted moieties of
known identity of the biological matter and function as a
marker for those molecules which are to be identified. For
example, when specific proteins is to be analyzed, anti-
bodies or fragments thereof, which are directed towards
30 specific targeted moieties on the same, can be used as
targeting molecules. Similarly, when a specific DNA-
sequence of a DNA-molecule is to be analyzed, the targeting
molecule is a complementary DNA-sequence to the nucleotide
of interest. The targeting molecule can also comprise a
35 chemical label, for example an unusual element or an

isotope, in order to improve the detectability in the analytical technique employed, i.e. when larger molecules (e.g. whole proteins) are to be detected. In this connection an unusual element or isotope means an element or an isotope which is not naturally present or present only in low concentrations in the biological matter analyzed.

The first step in the inventive method is to supply a sample of the biological matter as a specimen surface. Such a sample can be supplied as a specimen of a solid or semi-solid material. For example, an *in situ* specimen surface can be used directly, when the healing (ingrowth) of a titanium implant with a structured surface is to be studied.

The method according to the invention is general and can be used directly on complicated specimens, such as for example dialysis membranes after use. For example, peritoneal cells can be analyzed in connection with peritoneal dialysis by supplying the membrane used as specimen surface. Thus, the method is especially useful for studying cell preparations of blood cells on biomaterials. By studying material/blood reactions, information regarding the influence of man-made synthetic products on the cells can be detected directly.

The sample of the biological matter can also be supplied as a specimen surface by applying it on a solid surface, the solid surface being provided as a support for the biological matter. In this case the biological matter is in a more liquid state, such as blood and tissue fluid, but can also be a more delicate matter, such as a frozen tissue section.

The solid surface is generally a glass surface, but can be any other suitable solid surface in dependence on the specific application. This is especially relevant when cells are to be analyzed for adhesion, spreading or chemotactic movement.

If necessary, the specimen surface is also prepared by subjecting it to lyophilization, freeze-substitution, or air drying.

According to the invention, an imprint of the specimen surface is then produced on a substrate surface, whereby at least one chemical substance is distributed on the same. In order to be compatible with the analytical technique employed in the inventive method, the substrate surface should be a metal surface. Suitable metal substrate surfaces are silver, gold, palladium, platinum, nickel, chromium, and copper.

In order to improve the imprinting effect, the specimen surface should be pretreated immediately before the imprint is produced. One pretreatment comprises the condensation of liquid of a non-polar solvent and/or a polar solvent onto the specimen surface. Preferably, the polar solvent is a water solution.

The pretreatment can be accomplished by first bringing the specimen surface to room temperature or cooling the same to a lower temperature and then condensing the solvent vapor thereon by arranging the specimen above a heated container containing the liquid. The imprint should be produced within 10 s after the pretreatment of the specimen surface.

Likewise, the substrate surface should be polished and/or cleaned immediately before the imprint is produced. Suitable cleaning methods are chemical etching, plasma cleaning, and vaporization deposition. Of course, the cleaning methods can be combined.

Thus, a crucial step in the inventive method is the production of the imprint of the specimen surface on the substrate surface in order to distribute and "immobilize" chemical substance(s) on the same.

The imprint is preferably produced by pressing the specimen surface against the substrate surface. This can be

accomplished by pressing a compressible material against the opposite side of the specimen surface and/or the opposite side of the substrate surface and by applying thereon a force between 0.01 and 10 MPa. The pressing should be performed for up to 100 s.

In this process individual components, such as ions and larger molecules, are transferred to the substrate surface. An imprint of the distribution is obtained, which is dependent on the pretreatment and pressing parameters.

The pressing procedure is facilitated by the specimen surface and/or the substrate surface being made of a flexible material.

Likewise, the transfer of chemical substance(s) to the substrate surface is facilitated by the substrate surface being structured. Preferably, the substrate surface is structured with protrusions of 0.01-5 μm in width and/or length.

According to the invention, the imprint is then subjected to imaging mass spectrometry, wherein at least one signal is produced from at least one point of the substrate surface. The magnitude of this signal is dependent on the amount of the chemical substance present on the substrate surface.

The at least one signal is then recorded. Preferably, the signal is recorded from an array of points on the substrate surface.

Multiple sequential imprints can also be produced from the same area of the specimen surface. In this case each of the imprints is produced on a separate substrate surface. Then each imprint on each substrate surface is subjected to imaging mass spectrometry.

A suitable imaging mass spectrometry is a secondary ion mass spectrometry. Secondary ion mass spectrometry (SIMS) is a surface analytical technique that has been

employed for spatially resolved analysis of atoms and molecules at the single cell and subcellular levels.

In this connection silver is the preferred substrate surface, since silver is an almost optimal substrate for the analysis of intact molecular ions because of the ability of silver to cationize large molecules.

Thus, when deposited on a clean silver substrate the chemical substance(s) can be cationised by Ag^+ , peaks in the spectrum being provided which correspond to the mass of the intact molecule plus the Ag^+ ion $(M+\text{Ag})^+$. A conclusive identification of the detected molecules is then possible. The identification of unknown compounds is aided by spectral matchings with a library.

For the cationization of the chemical substance by substrate ions to occur in SIMS, the chemical substance to be analyzed must not be present on the substrate surface in too large quantities. Thus, the pressing is performed so that the imprint represents below 5 monolayers, preferably below 2 monolayers, of the chemical substance(s) on the substrate surface.

Preferably, a focused beam of ions should be produced by the primary ion source in the secondary ion mass spectrometry, the ions being C_{60} , Ga, In, or Au ions. When gold ions are used, they are clusters of n ions, in which $n \leq 10$. The focused beam should have a diameter below $10 \mu\text{m}$, preferably below $1 \mu\text{m}$.

The imaging mass spectrometry can also be a matrix assisted laser desorption ionisation. In this case a light sensitive matrix is applied onto the substrate surface before and/or after the production of the imprint. The light sensitive matrix can be α -cyano-4-hydroxycinnamic acid, trans-3-indoleacrylic acid, 3-methoxy-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, or 3,4-dihydroxycinnamic acid. The light source of the matrix

assisted laser desorption ionization should comprise a focused laser beam, preferably an ultraviolet laser beam.

At last the distribution of the at least one chemical substance is determined from at least one image of the
5 imaging mass spectrometry. Mass spectra are obtained with high mass resolution as well as images with high lateral resolution. The resolution is between 100 nm and 1 μ m.

Each image is in turn produced from the signal, the colour or the brightness in each point of the image being
10 dependent on the magnitude of the signal from the corresponding point on the substrate surface. In this way images of chemical distributions are obtained. The analysis as well as the regeneration of images is accomplished by means of advanced information technology, whereby image
15 processing as well as statistics for handling and processing of the large amounts of data is provided.

Preferably, the secondary ion mass spectrometry is time-of-flight secondary ion mass spectrometry (TOF-SIMS). This is a mass spectrometric method with a high lateral
20 resolution of down to 60 nm combined with the ability to measure secondary ions with masses up to at least 10 000 atomic mass units.

This type of secondary ion mass spectrometry is a relatively new technique for chemical surface analysis and
25 it has several advantages compared to other surface analysis methods. Most significantly, TOF-SIMS is the only method which has the potential for spatially resolved identification and chemical analysis of organic molecules on surfaces in the submicrometer range.

30 A TOF-SIMS spectrum is recorded under high vacuum by scanning the primary ion beam over the area of interest on the substrate surface and acquiring a positive or negative mass spectrum of the ions leaving the surface.

EXAMPLES

The invention will now be further described and illustrated by reference to the following examples. It should be noted, however, that these examples should not be construed as limiting the invention in any way.

Example 1. Ion microscopy.

Ions or other molecules from dried specimens are transferred to and immobilized on a silver surface by carefully pressing a freshly etched silver foil onto the dried specimen surface.

For analysis of organic compounds according to the invention, the accumulated primary ion dose is kept below the so called static limit, which means that the analysis is completed before the analyzed surface has been significantly damaged by the primary ions. In a TOF-SIMS image, the brightness of each pixel reflects the signal intensity of a selected ion in that pixel. The recorded TOF-SIMS spectra are stored in raw data files which contain complete spatial and spectral information from the data collection, thereby allowing for subsequent extraction of images of arbitrary ions and extraction of mass spectra from restricted areas within the analysis area at any time after data collection. Data are collected at either high mass resolution $m/\Delta m > 7000$ or lateral resolution (< 100 nm).

Example 2. Whole blood in vitro.

Venous blood from a volunteer is sampled and placed in drops onto different material surfaces and incubated at 37°C in a humid chamber for varied periods of time. The coagulated blood is then gently washed off and the surfaces are allowed to dry in air. Each preparation is washed with distilled water and dried.

The result of this procedure is a surface layer of plasma proteins and blood cells. The blood cells adhere and

are activated differently at different surfaces by detecting the cell expression of integrins and selectins.

More specifically, capillary blood was placed in drops on a clean glass surface and incubated for 30 min at
5 37°C. The clot was rinsed off with Dulbeccos phosphate-buffered saline and the saline was removed from the glass surface-adhering cells by a rinse in 0.15M NH₄COOH at pH 7.2-7.4. The glasses were then placed on a solid copper block pre-cooled with liquid nitrogen in a vacuum chamber
10 that was evacuated down to 10⁻⁴-10⁻⁵ bar.

Example 3. Distribution of cell components.

A clean silver foil is pressed against a glass surface prepared as above and the imprinted silver foil is
15 subsequently analyzed by means of TOF-SIMS at different mass-to-charge ratios of different ions (m/z) with reference to Na⁺, K⁺, Ca⁺⁺, amino acids, and cholesterol, a resolution of less than 0.5 μm being obtained.

20 1) Distribution of m/z=23 (indicative of Na⁺).

In this case the resulting TOF-SIMS images showed platelets with a low internal concentration of Na⁺ and leukocytes (see below) with membrane leakage of Na⁺.

25 2) Distribution of m/z=30 (indicative of CH₄N⁺).

This signal is common for several different amino acids, their presence being established.

3) Distribution of m/z=39 (indicative of K⁺).

30 Platelets exhibit a high internal concentration of K⁺, indicating an intact membrane, whereas the leukocytes exhibit membrane leakage of K⁺.

4) Distribution of m/z=40.1 (indicative of Ca⁺⁺).

35 All cells exhibit a granular distribution of Ca⁺⁺.

5) Distribution of m/z=493.3 and m/z=495.3 (indicative of cholesterol-¹⁰⁷Ag⁺ and cholesterol-¹⁰⁹Ag⁺, respectively) and

m/z=879.6 and m/z=881.6 (indicative of cholesterol dimer-¹⁰⁷Ag⁺ and cholesterol dimer-¹⁰⁹Ag⁺, respectively),.

These combined distributions resulted in a very reliable localization of cholesterol in the cells studied.

5 Thus, the imaging of subcellular distribution can be demonstrated at a resolution better than 100nm for signals corresponding to Na⁺, K⁺, Ca⁺⁺, cholesterol and total protein.

10 *Example 4. Cell preparation.*

Three different cell preparation methods have been used, air drying, freeze substitution, and freeze drying. Air drying was performed in saline followed by rinsing with water or volatile buffers to remove salts. The presence of
15 salt always ruined all possibilities to obtain reproducible data. Freeze substitution was performed in ethanol, acid ethanol, methanol, acid methanol, methanol/water 80/20 in various combinations of buffers and volatile salts. The use of solvents, even dilute methanol, always removed cholesterol from the cytoplasmic membranes.
20

The only preparation method that gave reproducible localisation of membrane lipids was freeze drying in volatile salts. Cholesterol, cholesterol dimer and phosphocholine have been localised. Cholesterol and
25 phosphocoline showed different and apparently complementary localisation in surface-adhering leukocytes.

Example 5. Platelet adhesion and activation.

The adhesion and spreading of platelets on protein-coated surfaces is studied with respect to receptors
30 involved and membrane expression of integrins and selectin. The spreading of cells is often accompanied by changes in membrane composition e.g. the exposure of phosphatidylserine in the outer leaflet seen during apoptosis. Such an
35 exposure of other membrane lipids, also with a short

halftime due to extracellular breakdown, is studied by means of the method according to the invention.

Example 6. Chemotaxis.

5 Chemotaxis, defined as the ability of orientation and directed migration in chemical gradients, is a key response of the immune system and a universal cell biological phenomenon. The regulation of this process is complex and not characterized in detail. The compartmentalization of the
10 intracellular signaling system in chemotaxis is a key issue in understanding the mechanisms that control cell orientation in chemotactic gradients. The spatial intracellular resolution of the cell components provide data with reference to those mechanisms, especially to time resolution.
15 tion.

In this connection the inositol lipids (PIP2 and PIP3) are important lipid intracellular messengers under study that are involved in the local control of the actin cytoskeleton and they have distinct functions in the local
20 and global regulation of pseudopode formation. Other lipid mediators, such as diacyl glycerol, are involved in secretory responses, such as degranulation and superoxide release, are also studied.

Glass surfaces are first coated with different
25 proteins by means of physical adsorption, a routinely used technique. Freshly isolated cells are then incubated at the protein-coated surfaces. For experiments with chemotaxis, special chambers have been constructed for exposure of the cells with a gradient of a chemoattractant. Cell adhesion,
30 polarisation and spreading is studied by means of the method according to the invention and compared with fluorescence microscopy.

Accordingly, the inventive method ("ion microscopy") can be used as a tool in cell biology and enables the
35 analysis and localisation of cell signal mediators, like phospholipids, lipid oxidation products, and ultimately

large molecules like whole proteins. The global molecular distribution of individual components within a cell can be reproduced in order to obtain cell specific information on subcellular dynamics of gene expression and proteins.

- 5 The method is also applicable to cell surface interactions as well as the influence of different drugs on cell reactions. A comparison can be performed before and after the biological matter has been exposed to different environmental factors. In addition, new materials can be
- 10 studied, which are developed for the treatment of wounds, dialysis and implants.

1
2
3
4
5
6
7
8
9
10
11
12

CLAIMS

1. A method of analyzing the spatial distribution of at least one chemical substance retained by a biological matter, characterized by the steps of
- 5 (a) supplying a sample of said biological matter as a specimen surface;
- (b) producing an imprint of said specimen surface on a substrate surface, said at least one chemical substance
- 10 being distributed on the same;
- (c) subjecting said imprint to imaging mass spectrometry, at least one signal from at least one point of said substrate surface being produced, the magnitude of said at least one signal being dependent on the amount of said at
- 15 least one chemical substance present on said substrate surface;
- (d) recording said at least one signal; and
- (e) determining said distribution of said at least one chemical substance from at least one image of said imaging
- 20 mass spectrometry.
2. The method as in claim 1, wherein said at least one chemical substance mainly comprises organic material.
3. The method as in claim 2, wherein said organic material comprises a lipid, an amino acid, a peptide, a
- 25 protein, a carbohydrate, a nucleotide, a transmitter substance, a drug, or a targeting molecule.
4. The method as in claim 3, wherein said nucleotide is a DNA-molecule.
5. The method as in claim 3 and 4, wherein said
- 30 targeting molecule is a complementary DNA-sequence.
6. The method as in claim 3, wherein said targeting molecule is an antibody or a fragment thereof.
7. The method as in any of claims 3-6, wherein said targeting molecule comprises a chemical label.
- 35 8. The method as in claim 7, wherein said chemical label is an unusual element or an isotope.

9. The method as in any of claims 1-8, wherein said biological matter comprises cells, tissue, virus, body liquid, or biological molecules.

10. The method as in any of claims 1-9, wherein said sample of said biological matter is supplied as a specimen surface *in situ*.

11. The method as in any of claims 1-9, wherein said sample of said biological matter is supplied as a specimen surface by applying it on a solid surface.

12. The method as in claim 11, wherein said solid surface is a glass surface.

13. The method as in any of claims 1-12, wherein multiple sequential imprints are produced from the same area of said specimen surface, each of said imprints being produced on a separate substrate surface.

14. The method as in any of claims 1-13, wherein said specimen surface is pretreated immediately before said imprint is produced.

15. The method as in claim 14, wherein said specimen surface is pretreated by condensing a liquid of a non-polar solvent and/or a polar solvent onto the same.

16. The method as in claim 15, wherein said polar solvent is a water solution.

17. The method as in claim 15 or 16, wherein said specimen surface is first brought to room temperature or cooled and is then arranged above a heated container containing said liquid.

18. The method as in any of claims 14-17, wherein said imprint is produced within 10 s after said pretreatment of said specimen surface.

19. The method as in any of claims 1-18, wherein said specimen surface and/or said substrate surface is made of a flexible material.

20. The method as in any of claims 1-19, wherein said substrate surface is a metal surface.

21. The method as in claim 20, wherein said metal is silver, gold, palladium, platinum, nickel, chromium, or copper, preferably silver.

22. The method as in any of claims 1-21, wherein said
5 substrate surface is structured.

23. The method as in claim 22, wherein said substrate surface is structured with protrusions of 0.01-5 μm .

24. The method as in any of claims 1-21, wherein said substrate surface is polished.

10 25. The method as in any of claims 1-24, wherein said substrate surface is cleaned immediately before said imprint is produced.

26. The method as in claim 25, wherein said substrate surface is cleaned by means of chemical etching, plasma
15 cleaning, or vaporization deposition, or a combination thereof.

27. The method as in any of claims 1-26, wherein said specimen surface is subjected to lyophilization, freeze-substitution, or air drying before said imprint is
20 produced.

28. The method as in any of claims 1-27, wherein said imprint is produced by pressing said specimen surface against said substrate surface.

29. The method as in claim 28, wherein said pressing
25 is accomplished by means of a compressible material.

30. The method as in claim 28 or 29, wherein said pressing is accomplished by applying a force between 0.01 and 10 MPa.

31. The method as in any of claims 28-30, wherein
30 said pressing is performed for up to 100 s.

32. The method as in any of claims 1-31, wherein said said pressing is performed so that said imprint represents below 5 monolayers, preferably below 2 monolayers, of said at least one chemical substance on said substrate surface.

33. The method as in any of claims 1-32, wherein said imaging mass spectrometry is a secondary ion mass spectrometry.

34. The method as in claim 33, wherein said secondary ion mass spectrometry is time-of-flight secondary ion mass spectrometry.

35. The method as in claim 33-34, wherein a focused beam of ions is produced by the primary ion source in said secondary ion mass spectrometry.

36. The method as in claim 35, wherein said ions are C₆₀, Ga, In, or Au ions.

37. The method as in claim 36, wherein said Au ions are clusters of n ions, $n \leq 10$.

38. The method as in any of claims 35-37, wherein said focused beam has a diameter below 10 μm , preferably below 1 μm .

39. The method as in any of claims 1-32, wherein a light sensitive matrix is applied onto said substrate surface before said imprint is produced.

40. The method as in any of claims 1-32 and 39, wherein a light sensitive matrix is applied onto said substrate surface after said imprint is produced.

41. The method as in any of claims 1-32 and 39-40, wherein said imaging mass spectrometry is matrix assisted laser desorption ionisation.

42. The method as in claim 41, wherein the light source of said matrix assisted laser desorption ionization comprises a focused laser beam, preferably an ultraviolet laser beam.

43. The method as in any of claims 1-42, wherein said at least one signal is recorded from an array of points on said substrate surface.

44. The method as in any of claims 1-43, wherein said at least one image is produced from said at least one signal, the colour or the brightness in each point of said

at least one image being dependent on the magnitude of said
at least one signal from the corresponding point on said
substrate surface.

1
2
3
4
5
6
7
8
9
0

ABSTRACT

A method of analyzing the spatial distribution of at least one chemical substance retained by a biological matter comprises the steps of

- (a) supplying a sample of said biological matter as a specimen surface;
- (b) producing an imprint of said specimen surface on a substrate surface, said at least one chemical substance being distributed on the same;
- (c) subjecting said imprint to imaging mass spectrometry, at least one signal from at least one point of said substrate surface being produced, the magnitude of said at least one signal being dependent on the amount of said at least one chemical substance present on said substrate surface;
- (d) recording said at least one signal; and
- (e) determining said distribution of said at least one chemical substance from at least one image of said imaging mass spectrometry.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183